

Diabetic Mouse Angiopathy Is Linked to Progressive Sympathetic Receptor Deletion Coupled to an Enhanced Caveolin-1 Expression

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Objective—Clinical studies have demonstrated that hyperglycaemia represents a major risk factor in the development of the endothelial impairment in diabetes, which is the first step in vascular dysfunction. Using non-obese diabetic mice, we have evaluated the role of the adrenergic system and eNOS on progression of the disease

Methods and Results—When glycosuria is high (20 to 500 mg/dL), there is a selective reduction in the response to α_1 and β_2 agonists but not to dopamine or serotonin. When glycosuria is severe (500 to 1000 mg/dL), there is a complete ablation of the contracture response to the α_1 receptor agonist stimulation and a marked reduced response to β_2 agonist stimulation. This effect is coupled with a reduced expression of α_1 and β_2 receptors, which is caused by an inhibition at transcriptional level as demonstrated by RT-PCR. In the severe glycosuria (500 to 1000 mg/dL), although eNOS expression is unchanged, caveolin-1 expression is significantly enhanced, indicating that high glucose plasma levels cause an upregulation of the eNOS endogenous inhibitory tone. These latter results correlate with functional data showing that in severe glycosuria, there is a significant reduction in acetylcholine-induced vasodilatation.

Conclusions—Our results show that in diabetes development, there is a progressive selective downregulation of the α_1 and β_2 receptors. At the same time, there is an increased expression of caveolin-1, the endogenous eNOS inhibitory protein. Thus, caveolin-1 could represent a new possible therapeutic target in vascular impairment associated with diabetes. (*Arterioscler Thromb Vasc Biol.* 2004;24:721-726.)

Key Words: eNOS ■ caveolin-1 ■ adrenergic system ■ vascular impairment ■ diabetes

It is well established that the appearance of cardiovascular disorders is much more frequent and severe in diabetic than in healthy subjects.^{1,2} Clinical studies have shown that hyperglycaemia represents a major risk factor in the development of the endothelial impairment, which is the first step in vascular dysfunction.³⁻⁷ Up to 80% of deaths in patients with diabetes are closely associated with vascular diseases,⁸ including coronary atherosclerosis, macroangiopathy, autonomic neuropathy, and diabetic cardiomyopathy.^{9,10}

Type 1 diabetes, or insulin-dependent diabetes mellitus (IDDM), is an autoimmune disease characterized by a islet inflammation or insulinitis, followed by progressive destruction of pancreatic β cells, followed by insulin secretion deficiency resulting in hyperglycaemia.^{11,12} The majority of current knowledge on the mechanisms involved in the hyperglycaemia-induced endothelial dysfunction has been obtained from vessels harvested from streptozocin-induced or alloxan-induced diabetic rabbits,^{13,14} rats,¹⁵⁻¹⁷ and mice.^{18,19} It has to be considered that these widely used animal models

rely on a rapid and extensive Langerhans islet β cell damage, characterized by the onset of high-level glycemia. This rapid shift from normal to high glycemia levels represents a critical limitation for these experimental models of IDDM because the appearance of hyperglycemia is not gradual as it is in humans. An alternative experimental approach is represented by non-obese diabetic mice (NOD/Ltj), a strain that spontaneously has autoimmune diabetes development with remarkable analogy to human IDDM.^{20,21} The disease is characterized by lymphocyte infiltration into the pancreatic islets, which progressively induces pancreatic β cell necrosis leading to IDDM.²² Diabetes develops gradually in mice and its onset is \approx week 15. Thus, this mouse strain represents an elective tool in investigating the vascular complications linked to diabetes development, because it is possible to follow-up the disease from its onset as well as during its progression. The relation between the disease progression and the onset of the vascular impairment has never been investigated in details. By using NOD mice, we have investigated on

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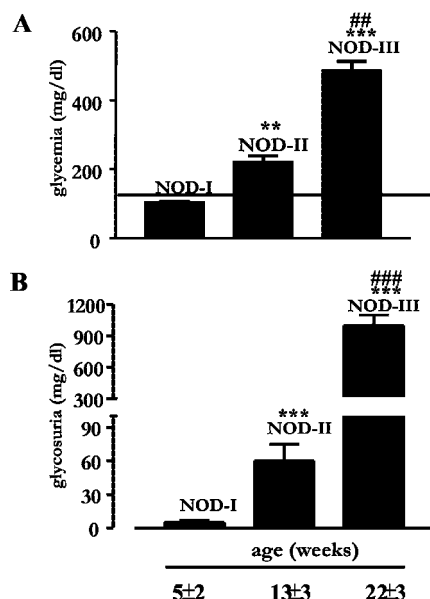


Figure 1. Correspondence between the NOD mice age and value of glycemia or glycosuria. Glycemia (A) and glycosuria (B) are elevated in an age-dependent fashion. The continuous line (A) indicates average glucose blood value in normoglycemic mice. The content of glucose in urine and plasma was measured by using calorimetric reaction. ** $P < 0.01$, *** $P < 0.001$ versus NOD I; ### $P < 0.01$, ### $P < 0.001$ versus NOD II. Data are presented as mean \pm SEM, $n = 12$ for each group.

the role of sympathetic system by using aortas isolated from mice with different levels of glycosuria. In addition, we have also evaluated the involvement of eNOS and of its endogenous regulatory protein, caveolin-1 (CAV-1).

Methods

Phenylephrine-Induced Vasoconstriction Is Reduced in NOD Aortic Rings and Is Closely Related to Glycosuria

To investigate whether diabetes causes changes in vascular reactivity, phenylephrine (PE)-induced cumulative concentration-response curve was performed in control and NOD mice that were divided in 3 groups according to glycosuria/glycemia levels (Figure 1). Because NOD group 1 mice demonstrated a similar pattern of response as did CD-1 mice, indicating that at this stage of the disease no pathological changes occurred, CD-1 and NOD group 1 mice have been considered as normal control response. In NOD groups 2 and 3, the PE-induced cumulative concentration-response curve was significantly reduced when compared with NOD group 1 and CD-1 mouse aortic rings (Figure 2A). In particular, PE-induced contractions in NOD group 2 were significantly reduced compared with NOD group 1 and CD-1 mouse aortic rings. In addition, NOD group 3 PE-induced contractions were abolished. Conversely, 5-HT-induced (Figure 1, available online at <http://atvb.ahajournals.org>; 5-HT [A] and DA [B] on aortic rings harvested from NOD mice. 5-HT-induced and DA-induced cumulative concentration-response curves [10 nM to 30 μ M/L] were not significantly different in NODs and CD-1 mice. SNP-induced [C] cumulative concentration-response curves showed no significant difference between NOD groups 1, 3, and CD-1 mice. Data are presented as mean \pm SEM of percent of vasorelaxation, $n = 8$ for each group) and DA-induced cumulative concentration-response curves were not significantly different between aortic rings from all NOD mouse groups and were similar to CD-1 mouse aortic rings. These observations showed that with

diabetes progression, as assessed by glycosuria, NOD mice have a selective deficit to α_1 adrenergic stimulation.

Isop-Induced Vasorelaxation Is Reduced in NOD Aortic Rings and Is Closely Related to Glycosuria

Isop-induced cumulative-concentration response curve was performed to investigate whether the reduced response of α_1 adrenergic receptors was shared with β_2 adrenergic receptors. As shown in Figure 2C, Isop-induced cumulative concentration-response curve was significantly reduced compared with NOD group 1 and CD-1 mice aortic rings. In particular, Isop-induced vasorelaxation in NOD group 2 results were significantly curtailed when compared with NOD group 1 and CD-1 mice aortic rings. Again, in NOD group 3, there was a major effect and Isop-induced vasorelaxation was markedly reduced. Thus, with diabetes progression, NOD mice appear to be less responsive to β_2 adrenergic stimulation.

RT-PCR and Western Blot Studies

The effects observed in the functional experiments suggested hypofunctionality of α_1 and β_2 adrenergic receptors or a reduction in number of the adrenergic receptors. To further verify this hypothesis, we performed a Western blot analysis on NOD groups 1, 2, 3, and CD-1 aortas for α_1 adrenergic receptor. As shown in Figure 3, α_1 (Figure 3A) and β_2 (Figure 3B) receptor expression was reduced. This reduction was related to diabetes progression, with a remarkable reduction of receptor expression in NOD group 3 compared with NOD group 1 and CD-1 aortas (Figure 3A and 3B). These results reinforce the finding that with diabetes progression, an impairment of adrenergic function occurs in α_1 -mediated vasoconstriction and β_2 -mediated vasorelaxation caused by a reduction of receptor expression. This view was further confirmed by the RT-PCR experiment (Figure 3C). PCR analysis demonstrated a significantly decreased α_1 and β_2 mRNA expression in NOD 3 aortas in comparison with NOD 1 mice, indicating that the reduction of receptor expression was caused by an inhibition of their transcription.

Results

Ach-Induced, but not SNP-Induced Vasorelaxation Is Reduced in NOD Aortic Rings

Ach causes an endothelium nitric oxide (NO)-dependent relaxation. In NOD group 3, the Ach-induced cumulative concentration response curve was significantly reduced compared with NOD groups 1, 2, and CD-1 mouse aortic rings (Figure 2D). Because PE-induced contraction in NOD groups 2 and 3, mouse aortic rings were weaker (see previous) when compared with CD-1 mice. Ach cumulative concentration-response curve was performed on 5-HT precontracted rings (Figure 2D). To verify the integrity of smooth muscle, SNP-induced vasorelaxation (NO donor) was also investigated. SNP-induced cumulative concentration-response curves showed no significant difference among aortic rings from all NOD groups and CD-1 mice (Figure 1), indicating that diabetes does not directly influence smooth muscle relaxation mediated by NO. To define the involvement of basal NO release in Ach-induced vasorelaxation, the tonic tone was removed by using a NO inhibitor. Increase in tension development generated by the NO synthase inhibitor L-NAME (100 μ M/L) on 5-HT precontracted rings obtained from NOD groups 2 and 3 mice was significantly reduced compared with NOD group 1 and CD-1 mice (Figure 2B). This effect is more consistent in NOD group 3 aortic rings, suggesting that severe diabetic conditions result in an impairment of basal NO release.

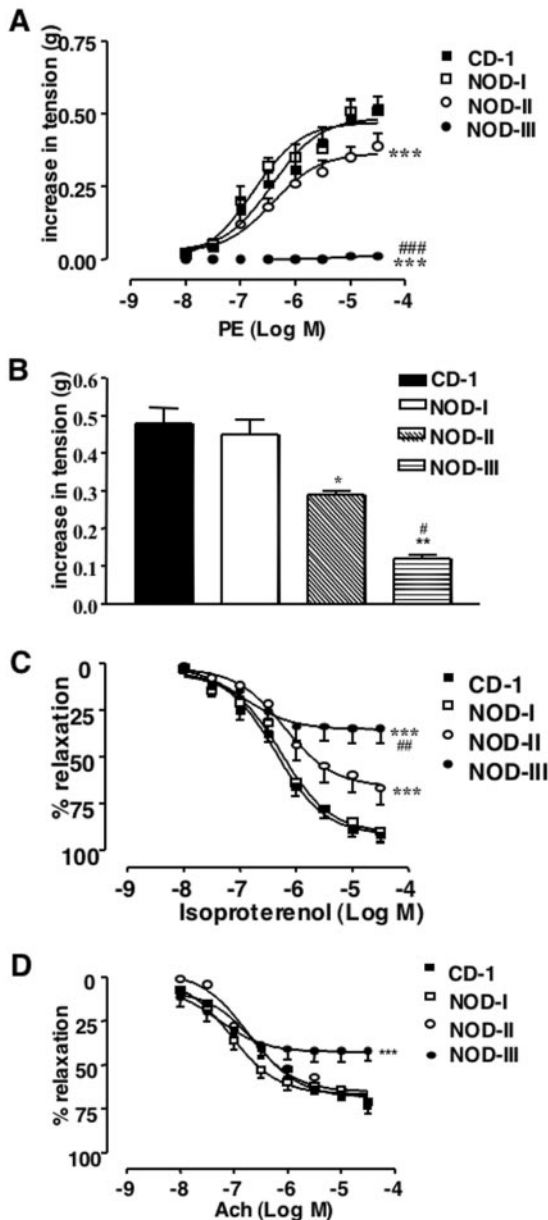


Figure 2. A, PE-induced cumulative concentration-response curve (10 nM to 30 μ M) was reduced in NOD group 2 (*** P < 0.001) compared with NOD group 1 and CD-1 mice. In NOD group 3, PE-induced vasoconstriction is virtually absent (*** P < 0.001 versus NOD group 1 and CD-1 mice; ### P < 0.001 versus NOD group 2). Effects of L-NAME-induced (B) increases in isometric tension were progressively reduced in NOD groups 2 (* P < 0.05) and 3 (** P < 0.01, # P < 0.05 versus NOD group 2) compared with NOD group 1 and CD-1 mice. Data are presented as mean \pm SE of the increase of tension, n = 6 for each group. C, Isop-induced cumulative concentration-response curve (10 nM to 30 μ M) was progressively reduced in NOD groups 2 and 3 compared with NOD group 1 and CD-1 mice. Isop-induced vasorelaxation in NOD group 2 was significantly diminished when compared with NOD group 1 and CD-1 mice (*** P < 0.001). Reduced vasorelaxation was even more pronounced in NOD group 3 mice (*** P < 0.001 versus NOD group 1 and CD-1 mice; ### P < 0.01 versus NOD group 2). Data are presented as mean \pm SEM of the increase of tension, n = 8 for each group. D, Ach-induced cumulative concentration-response curve was significantly reduced in NOD group 3 compared with NOD groups 1, 2, and CD-1 mice (*** P < 0.001). In this protocol, aortic rings were precontracted with 5-HT because PE-induced contraction was weaker (see Figure 2) in NOD groups 2 and 3 compared with NOD group 1 and CD-1 mice.

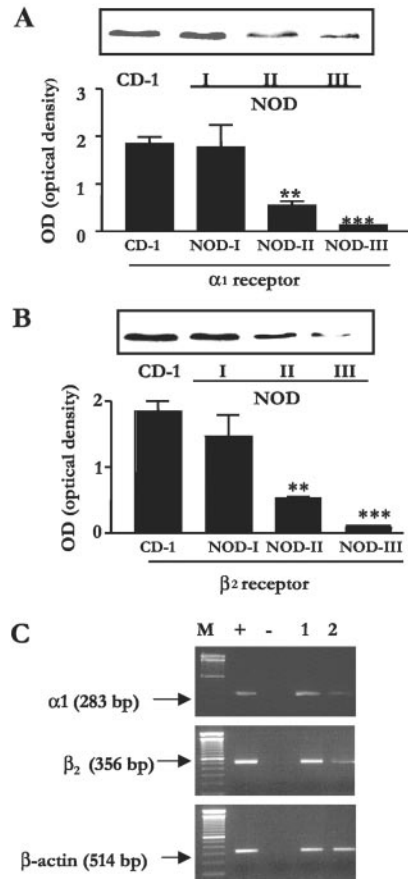


Figure 3. A, Densitometric analysis shows that there is a remarkable reduction of α_1 adrenergic receptor expression in NOD groups 2 and 3 compared with NOD group 1 and CD-1 aortas. The blot shown is representative of 3 separate experiments. ** P < 0.01; *** P < 0.001 versus CD-1 and NOD-1. B, Western blot analysis on NOD groups 1, 2, 3 and CD-1 aortas for β_2 adrenergic receptor. Densitometric analysis shows that there is a remarkable reduction of β_2 adrenergic receptor expression in NOD group 3 compared with NOD group 1 and CD-1 aortas. The blot shown is representative of 3 separate experiments. ** P < 0.01, *** P < 0.001 versus CD-1 and NOD 1. C, mRNA expression of α_1 and β_2 receptors is reduced in NOD 3 mouse aorta. M indicates molecular masses; -, negative control (water); +, positive control; 1, NOD 1 mouse aorta; 2, NOD 2 mouse aorta. The RT-PCR shown is representative of 3 separate experiments.

Basal Release of NO, eNOS, and Caveolin-1 Involvement in NOD Aortic Rings

To investigate if the reduction of Ach-induced vasorelaxation was related to a reduction in eNOS expression, we performed Western blot analysis of eNOS expression on NOD groups 1, 2, 3, and CD-1 aortas. As shown in Figure 4A, there was no difference of eNOS expression in the NOD group 3 compared with the NOD groups 1, 2, and CD-1 aortas, whereas caveolin-1 expression was significantly enhanced in NOD group 3 mice (Figure 4B and 4C). This result suggests that the impairment of Ach-induced vasorelaxation in NODs is not caused by a reduced eNOS expression but by an enhanced negative modulation of eNOS by caveolin-1.

Cell Experiments with Normal and High Glucose Environment

To test the effect of high glucose on eNOS activity (eg, NO production), we used 2 cell lines, BAEC and HEK293, stable

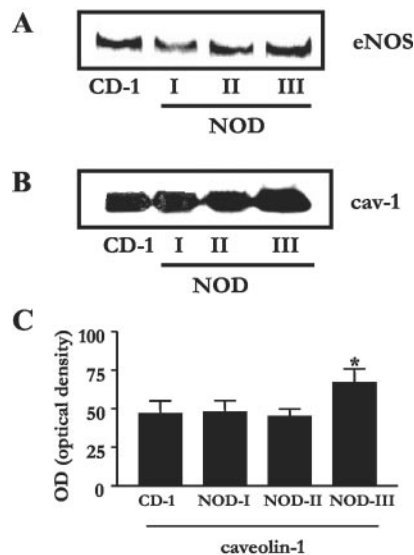


Figure 4. A, Disease progression does not affect eNOS expression. B, The disease progression significantly increases caveolin-1 expression. Blots are representative of 3 separate experiments. C, Densitometric analysis shows that there is a significant increase in CAV-1 expression in NOD group 3 mice only. * $P < 0.05$ versus CD-1, NOD groups 1 and 2.

transfected with eNOS.²⁴ BAEC in normal glucose environment produces a basal release of NO of ≈ 2 nM of nitrite, whereas on stimulation with calcium ionophore A-23187 nitrite levels shift to ≈ 13 nM with a 6-fold increase in the response. When cells are cultured in high glucose levels, the basal level of NO release was not significantly affected whereas the stimulated release by A-23187 was significantly reduced by $\approx 50\%$ (Figure 5A). This reduced NO release correlates with the increase in caveolin-1 expression in BAEC (Figure 5B). In the same experimental setting, NOS activity was determined by monitoring conversion of labeled arginine in citrulline. Treatment of BAEC with calcium ionophore A-23187 (10^{-5} M) in high-glucose environment caused a reduction of NOS activity of $\approx 70\%$, shifting the conversion of L-(3 H)-arginine to L-(3 H)-citrulline from 247 ± 47 pmol/min per milligram of protein to 65 ± 57 pmol/min per milligram of protein. These results further confirm that the effect observed in high-glucose environment in stimulated conditions is related to a reduction in eNOS activity rather than other mechanisms such as NO quenching. Next, to further demonstrate that the reduced NO release was caused by glucose effect on eNOS, we used HEK293 stably transfected with eNOS. As expected in these cells, the basal release of NO was higher and it was significantly increased by treatment with A-23187. When HEK293 were cultured in high-glucose environment, there was a marked reduction in NO basal production as well as in the stimulated condition (eg, A-23187-stimulated), thereby establishing that high-glucose environment negatively modulates eNOS activity (Figure 5C).

Discussion

Szentivanyi and Pek first showed an altered vasodilatory response in IDDM patients in the arterial bed of the conjunctiva in 1973.²³ Since then, in several studies it has been demonstrated that a substantial link between diabetes and vascular disorders

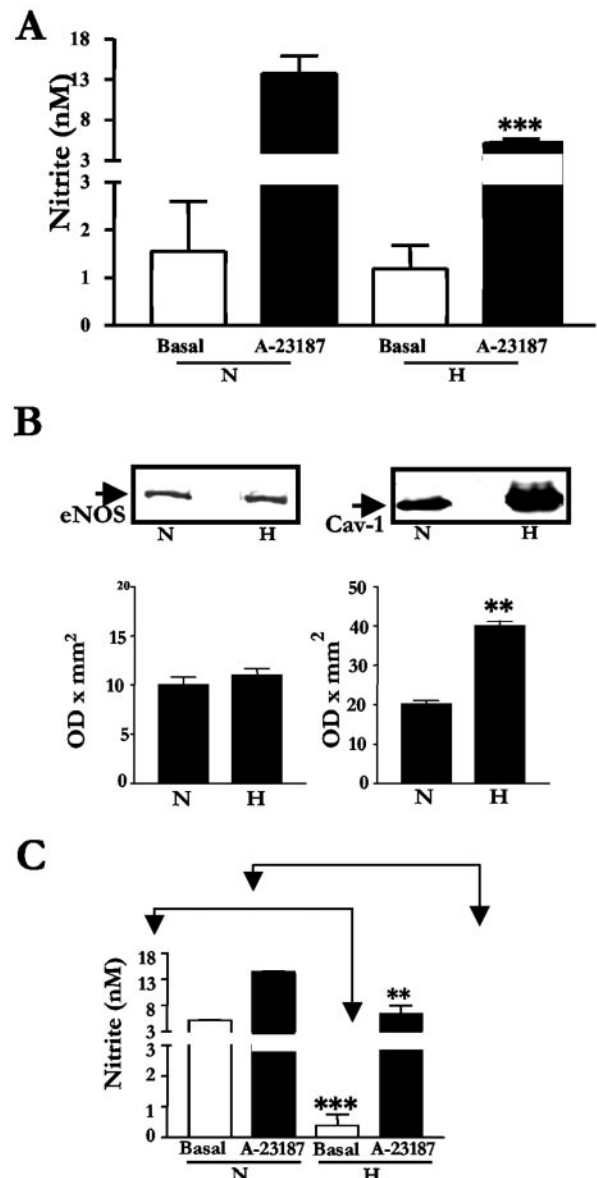


Figure 5. A, In BAEC exposed to high-glucose environment, there is a significant reduction of A-23187-induced NO release ($n=4$; $P < 0.001$). B, Reduction in NO release is coupled to an increased caveolin-1 expression ($n=4$; $P < 0.01$) with no changes in eNOS expression ($n=4$; NS). C, HEK-293 cells stably transfected with eNOS in the basal ($n=4$; $P < 0.01$) and the stimulated release of NO ($n=4$; $P < 0.001$) are significantly reduced in high-glucose environment. ** $P < 0.01$ stimulated in high-glucose (H) versus stimulated in normal glucose (N); *** $P < 0.001$ basal in H versus basal in N. The results are expressed as mean \pm SEM of 4 separate experiments

culminate in atherosclerosis, with the most common complication being diabetes.^{24–28} In the past 10 years, much attention has been focused on a particular strain of mice whose diabetes strongly resembles the human disease (the NOD mice).²⁹ However, these mice have been used to study diabetes on elevation of blood glucose over the physiological limit at different pathological values of glycemia or glycosuria and were selected for specific different experimental protocols. Thus, most of the results are not comparable, and studies on the linkage between diabetes progression and vascular functionality on this particular strain of mice are still lacking.

Vascular tone is driven by a dynamic process in which several signals that are either cell-mediated or receptor-mediated are involved. A major role is played by vascular adrenoreceptors, and this effect is coupled to the regulatory role played by the endothelium through mediator release. This complex and finely tuned interplay is disrupted by diabetes resulting in endothelial dysfunction. Our study shows that NOD group 1 mice, with null or low glycosuria, behave as normal outbred CD-1 mice. This result implies that it is important to use NOD mice with consistent glycosuria/glycemia to study angiopathy associated with diabetes. Indeed, in NOD mice with high glycosuria (NOD group 2 and group 3), an impaired vascular reactivity of the adrenergic system was consistently observed. In particular, high glycosuria (NOD group 2 mice) is associated with a reduction in α_1 receptor expression, whereas a severe glycosuria (NOD group 3 mice) causes a complete ablation of the contractile response associated with a further reduction of α_1 receptor expression. Similarly, the relaxing response to isoproterenol (β_2) is reduced in high and severe glycosuria associated with a reduction in β_2 receptor expression. PCR analysis performed on NOD group 3 mice aortas demonstrated that high glucose levels downregulated both receptors at transcriptional level. This experimental evidence finds a match with the human disease, in which it has been shown that in non-complicated IDDM there is a chronic decrease in sympathetic drive.³⁰ In addition, it has been shown that insulin infusion increases sympathetic activity,³¹ suggesting that the lack of insulin in diabetes type 1 in humans is directly related to a reduction of the adrenergic function. Thus, it appears that diabetes progression is linked to a selective disruption of the sympathetic drive. To evaluate if in this animal model there is such selectivity, we have assessed if vascular reactivity impairment in NOD mice is specific for adrenergic receptors by using another 2 contracture agents, eg, 5-HT and DA. Aortic rings from NOD group 3, in which there is a complete lack of response to α_1 stimulation, still contracted to 5-HT and DA, further stressing that diabetic conditions caused a selective impairment of the adrenergic function. It is also important to note that NOD group 2, in which glycosuria ranges between 20 mg/dL and 500 mg/dL, displays the same impaired response to adrenergic stimulation and reduced expression of α_1 and β_2 receptors.

Another important role in controlling vascular homeostasis is played by eNOS-derived NO. Thus, we have investigated the role of endothelium by measuring NO-dependent response in the aortic rings. A significant reduction in Ach-induced vasorelaxation was observed in mice with severe glycosuria, whereas at all the other levels of glycosuria the response to Ach was unchanged. Aortas from NOD 1, 2, and 3 relaxed similarly with exogenous NO. These results imply that L-arginine/NO pathway is the last to be impaired by the progression of the disease; and vessels, even at very high levels of glycosuria, still maintain at least in part their ability to relax with endogenous agonist, eg, Ach. It is known that vessels generate a tonic amount of NO that is dynamically and constantly produced by eNOS and that removal of this tonic activity by addition of an NO inhibitor causes an increase in vascular tone in vitro and blood pressure in vivo. When rings from NOD group 2 and 3 mice were challenged with L-NAME, there was a marked and significant reduction in the increase in tension indicating that in the

presence of high or severe glycosuria, an impairment of eNOS activation/activity occurs. Thus, in this mouse strain reduction in vascular adrenoreceptor expression is also coupled with a reduction in formation of eNOS-derived NO. Also, this finding fits with the demonstration that IDDM patients have an impairment in NO release.^{32–34} Western blot analysis of aortas of NOD groups and CD-1 demonstrated that there are no differences in eNOS expression, indicating that the reduction of NO release in diabetes is not linked to a reduced expression of the enzyme. This finding is further corroborated by the cell studies performed in high-glucose and normal glucose environments. Whereas in BAEC grown in high glucose levels basal NO release is not affected, a significant reduced production of NO in stimulated condition (eg, calcium ionophore A23917) is present. The finding that the high-glucose environment specifically modulates eNOS activity is further confirmed by the reduced conversion of labeled arginine in citrulline. In addition, in HEK 293 stably transfected with eNOS, there is a significant reduced production of NO in basal and stimulated conditions in the high-glucose environment. eNOS activity is regulated by dynamic subcellular targeting, regulatory protein–protein interactions,^{35,36} and protein phosphorylation,^{37,38} many of which can be modulated by stimuli in a calcium-dependent or calcium-independent manner.^{35,36} In general, in the basal state, eNOS is negatively regulated by several proteins, thus producing low levels of NO. eNOS is located within the plasma membrane microdomain caveolae, where it is complexed with the coat protein for this organelle, termed CAV-1. CAV-1 is a 22-kDa protein that decorates the cytoplasmic surface of caveolae and serves as the primary structural component of caveolae (50 to 100 nm invaginations of the plasma membrane), where it acts as a physiological inhibitor of eNOS.^{39,40} On activation of endothelial cells by a cadre of mediators, the caveolin-1 inhibitory clamp is diminished by the recruitment of several proteins that promote an activation complex.⁴¹ For this reason, we have evaluated whether diabetic conditions could modulate caveolin-1 expression downregulating through this mechanism of eNOS activity. Western blot analysis for caveolin-1 shows a significant increase of caveolin-1 expression in NOD group 3 aortas only, and this is in agreement with the functional data in which a significant reduction to Ach response has been found in NOD group 3 only. Furthermore, a similar increase in caveolin-1 coupled with an increase in NO production is present in BAEC cells in high-glucose environments. Thus, the reduction of NO release in IDDM can be ascribed to a reduction in eNOS activity through an enhanced expression of caveolin-1. This hypothesis is corroborated by a study in which it has been shown that in adipocyte plasma membrane, insulin receptor is localized in caveolae,⁴¹ the same microdomain where eNOS resides.⁴² In addition, it has been shown that caveolin-1, through its scaffolding domain, potently stimulated insulin receptor kinase activity, stabilizing the activated conformation of the regulatory region of the receptor in an activated state.⁴³ Thus, it is feasible that IDDM, in which a progressive reduction of insulin production occurs, there is an increase of caveolin-1 expression that leads eNOS activity inhibition that in turn reduces NO production and promotes and stabilizes insulin receptor in its active conformation. This latter finding could be interpreted as a body-adaptive response to a reduction in insulin production, in which insulin

receptor is more receptive to a minor concentration of insulin because of IDDM.

In conclusion, on progression of diabetes in NOD mice, there is a marked selective reduction in the adrenergic response associated with a reduction in the expression of α_1 and β_2 receptors on vessels. When glycosuria is severe (eg, NOD group 3), a reduction in NO-dependent vasorelaxation occurs and this is coupled with an enhanced caveolin-1 expression. Our results indicate that studies on cardiovascular complications in diabetes performed using NOD mice should be performed while accounting for disease progression in mice. In addition, our results suggest that caveolin-1 may represent a new possible therapeutic target in diabetes.

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Diabetic Mouse Angiopathy Is Linked to Progressive Sympathetic Receptor Deletion Coupled to an Enhanced Caveolin-1 Expression

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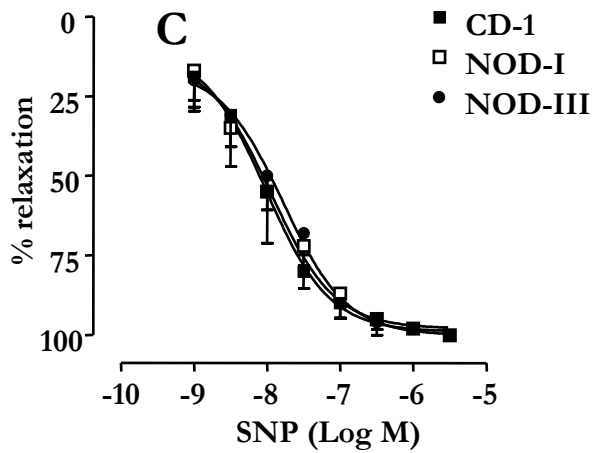
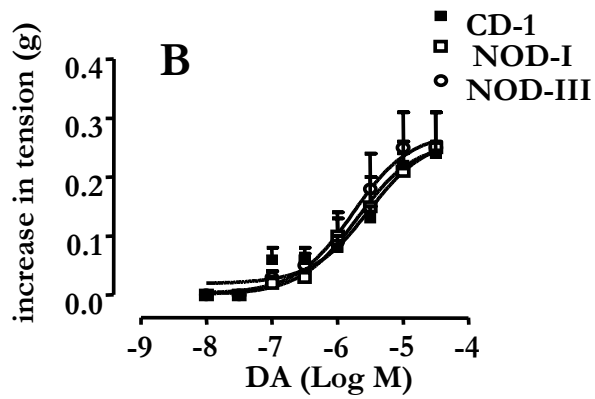
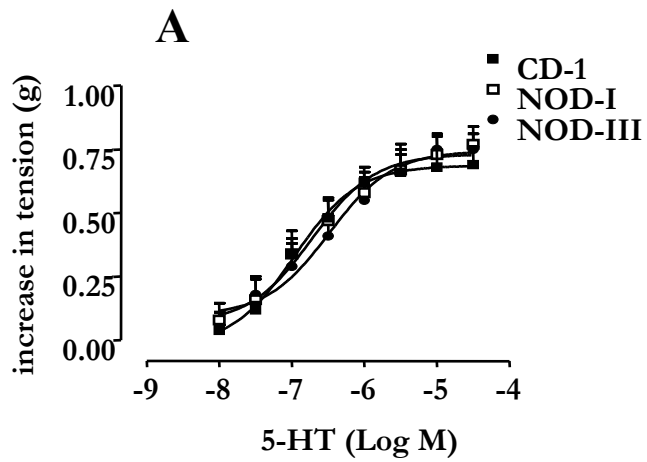
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Methods

Reagents

L-phenylephrine (PE), serotonin (5-HT), dopamine (DA), acetylcholine (Ach), isoproterenol (Isop), sodium nitroprusside (SNP), N ω -nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (Milano-Italy). All salts used for Krebs solution preparation were purchased from Carlo Erba Reagenti (Milan, Italy). Anti- α_1 , anti- β_2 and anti-caveolin-1 IgG were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). Anti-eNOS was purchased from Transduction Laboratories (BD Biosciences). All salts used for western blot analysis were purchased from ICN Biochemical (Eschwege, Germany).

Animals

Female Non Obese Diabetic mice (NOD/Ltj) and CD-1 mice, were purchased from Charles River (Italy). NOD mice exhibit a susceptibility to spontaneous development of autoimmune (type I) insulin dependent diabetes mellitus (IDDM)¹. Diabetes development in NOD mice is characterised by insulinitis and leukocytic infiltrate of the pancreatic islets. Progressive reduction in pancreatic insulin content starts at about 12-16 weeks of age.

Measurement of glycosuria

To assess the diabetic condition of NOD animals, glycosuria was evaluated weekly to select the animals. This method was used since it is non invasive and it well correlates with an increase in blood glucose. Groups of 3 mice were placed in metabolic cages able to selectively collect urine, for at least 4h. The content of glucose in the urine was measured by using Trinder reaction² (Glucose Trinder 100, Sigma Chemical Co. Milano, Italy). This method is based on the glucose oxidation to gluconic acid and hydrogen peroxide in the reaction catalysed by glucose oxidase. Briefly, the hydrogen peroxide formed reacts in the presence of peroxidase with 4-aminoantipyrine and p-hydroxybenzene sulfonate to form a quinoneimine dye, with maximum absorbance at 505 nm. The

value of absorbance is directly proportional to the glucose concentration in the sample. The value of glycosuria (gl) obtained is correlated to the age of the animals (Figure 1), therefore mice have been divided in the following groups:

- NOD group I $0 < \text{gl} < 5 \text{ mg/dl}$ = low or null glycosuria
- NOD group II $20 < \text{gl} < 500 \text{ mg/dl}$ = high glycosuria
- NOD group III $500 < \text{gl} \text{ to } 1000 \text{ mg/dl}$ = severe glycosuria

Animals were sacrificed at these different points and aortas were dissected and used for western blotting analysis or for tissue bath experiments. Experiments were performed also on CD-1 mice in order to have an internal non diabetic control to compare with NOD I

Tissue preparation

NOD or CD-1 mice were sacrificed and thoracic aorta was rapidly dissected and cleaned from fat and connective tissue. Rings of 1.5-2 mm length were cut and mounted on wire myographs (Kent Instruments, Japan) filled with gassed Krebs solution (95% O_2 + 5% CO_2) at 37°C. Changes in isometric tension were recorded with PowerLab data acquisition system (Ugo Basile, Italy). The composition of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, MgCl_2 0.0012, KH_2PO_4 0.0012, CaCl_2 0.0025, NaHCO_3 0.025 and glucose 0.010. Rings were initially stretched until a resting tension of 1.5 g was reached and allowed to equilibrate for at least 40 minutes during which tension was adjusted, when necessary, to 1.5 g and bathing solution was periodically changed. In a preliminary study a resting tension of 1.5 g was found to develop the optimal tension to stimulation with contracting agents.

Experimental protocols

In each experiment rings were firstly challenged with PE (1 μM) until the responses were reproducible. To evaluate tissue contractility, cumulative concentration response curve to PE (10 nM-30 μM), 5-HT (10 nM-30 μM) and DA (10 nM-30 μM) were performed. Conversely, to

evaluate tissue vasorelaxation, cumulative concentration response curve to Ach (10 nM-30 μ M), SNP (1 nM-3 μ M) and Isop (10 nM-30 μ M) were performed on PE-precontracted rings. In addition, Ach cumulative concentration response curve was performed also in 5-HT-precontracted rings. In a separate set of experiments rings were contracted with 5-HT 300 nM, once plateau was reached L-NAME 100 μ M was added.

Western Blotting

Aortic tissue samples were homogenised in lysis buffer (β -glycerophosphate 0.5 M, sodium orthovanadate 10mM, $MgCl_2$ 20mM, EGTA 10mM, DTT 100mM and protease inhibitors) using a Talon homogenizer, and were processed identically. Protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Segrate, MI). Proteins (30 μ g) were subjected to electrophoresis on an SDS 10% polyacrylamide gel and electrophoretically transferred onto a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). The immunoblots were developed with 1:500 dilutions for α_1 and β_2 receptors and 1:1000 for eNOS and caveolin-1, and the signal was detected with the ECL System according to the manufacturer's instructions (Amersham Pharmacia Biotech).

RT-PCR analysis

CD-1 and NOD mice were sacrificed and thoracic aorta was dissected and immediately snap frozen on liquid nitrogen. Total RNA was prepared using RNeasy Fibrous Tissue Kit (Qiagen) according to the manufacturer's instructions. Traces of genomic DNA that may copurify with RNA are removed by a DNase treatment on the same spin column provided with the kit. Total RNA was reverse-transcribed using random hexamers, as described. One-tenth of each cDNA sample was amplified by PCR with α_1 and β_2 adrenergic receptor-specific primers. Each sample contained the

sense and antisense primers 1 μ M, 200 μ M dNTPs, 50 mM KCl, 20 mM Tris- HCl, pH 8.6, 1.5 mM $MgCl_2$, and 1.25 U Platinum Taq Polymerase (Invitrogen). Thermal cycling was performed for 30 seconds at 94 °C, 30 seconds at 55°C and 45 seconds at 72°C After 24, 26 28 30 and 32 cycles 30 seconds pause were programmed to obtain 5 μ l sample. The sense and antisense primers (5'→3') were AGGCTGCTCAAGTTTTCCCG and GCTTGGAAGACTGCCTTCTG for α 1a adrenergic receptor (283 bp), and GCCATCCTCATGTCGGTTAT and AGCAGGCTCTGGTACTTGAA for β 2 adrenergic receptor (326 bp). For mouse β -actin the primers pair used were 5'-TGTGATGGTGGGAATGGGTCAG-3' for the sense and 5'-TTTGATGTCACGCACGATTTC-3' for the antisense sequence (514 bp). PCR was carried out for 30 cycles as follow: 45 seconds at 94 °C, 45 seconds at 60°C and 1 minute at 72°C (514 bp). Control PCR reactions also were performed on non- reverse transcribed RNA to exclude any contamination by genomic DNA. The amplified DNAs were analysed on a 1.8 % agarose gel, the fragment size was assessed by comparison with 100bp DNA marker (Invitrogen). The gel was photographed under ultraviolet transillumination with a Kodak Digital Science ID Image Analysis Software, images were the digitalised and a semi-quantitative analysis was performed using the same software.

Cell experiments with normal and high glucose environment

Human embryonic kidney (HEK-293) cells stable transfected with eNOS were gently provided by W.C. Sessa³. Bovine aortic endothelial cells BAEC cells were obtained by Istituto Nazionale per lo Studio e la Cura dei Tumori (Milano, Italy). The cells were cultured in 60mm Petri plastic dishes (FALCON, Microtech Italy) and grown in medium (GIBCO, Invitrogen Corporation) supplemented with 2mmol/l glutamine (GIBCO), 10% heat inactivated fetal calf serum (GIBCO), 50U/ml penicillin, 50U/ml streptomycin. The Petri dishes were incubated at 37°C in a 5%CO₂-95% air gas mixture. BAEC were subcultured on reaching confluence by use of 0.01% trypsin-EDTA. The cells were used between passage 5 and 6. Cells were grown until they reached 90% confluence and then were serum starved overnight.

BAEC were pre-treated for 3h with 11.5mM D-glucose solution (normal glucose) or 25mM D-glucose solution e. g. high glucose⁴ and eNOS activation by the known agonist the calcium ionophore A23187 (10^{-5} M, 30min)⁴ was examined. The medium was collected and nitrite were assayed fluorometrically in microtiter plates using a standard curve of sodium nitrite. The same protocol was utilised for HEK-293 reducing the time of incubation with high glucose to 2h. Preliminary experiments showed that high glucose induced apoptosis in Hek-293 cells after 3h.

eNOS activity

Activity was determined as described previously⁵ BAEC were incubated for 60 min with L- (3 H)-arginine 185×10^3 (5 μ Ci). Cells were washed twice with PBS in order to remove the L- (3 H)-arginine in excess. BAEC were then treated for 3h with 11.5mM D-glucose solution (normal glucose) or 25mM D-glucose solution and then calcium ionophore A23187 (10^{-5} M) added as described above. After 30 min of incubation 2ml of cold PBS containing 5mmol/L L-arginine and 4mmol/L EDTA were added to stop the reaction. Ethanol (0.4ml), 95%) was then added and after evaporation 2 ml of 20mmol/L Hepes –NA (pH 6) were added. The supernatants were collected, applied to 2 ml columns of Dowex AGWX8-200 (Aldrich, Milano, Italy) to trap radioactive arginine and eluted by 4ml of water to elute radioactive citrulline. Eluted radioactivity was measured by liquid scintillation counting.

Statistical Analysis

All data were expressed as mean \pm SEM. Statistical analysis was performed by using 2-way ANOVA and paired and unpaired Student t-test where appropriate. Differences were considered statistically significant when *P* value was less than 0.05.

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